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Isolation and Characterization of a Soil-Derived Microbial Strain from Gujarat with Potential Bioeconomic Applications

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Abstract: Plastics are low-cost, sturdy and versatile materials that can be considered valuable to humanity in many ways as they improve life quality and economic activity. Over the past two decades, the inherent durability and resistance to degradation that characterize plastic materials have increasingly been recognized as significant contributors to environmental pollution and waste-management challenges. Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible polymer that offers a sustainable alternative to synthetic plastics. Synthetically produced polymers are widely available and economical, but their extensive use has significant ecological consequences. As a result, millions of plastic products accumulate in the environment, contributing to increasing pollution. This situation highlights the urgent need to replace non-biodegradable plastics with naturally occurring, biodegradable alternatives. Polyhydroxybutyrate (PHB) is one such biodegradable polymer, synthesized by bacteria under environmental or nutritional stress and stored within the cells as a reserve material in the form of inclusion bodies. However, its widespread application is limited by high production costs and suboptimal properties. Soil samples from North Gujarat were used to isolate PHB-producing bacteria through serial dilution and plating on LB agar (Luria-Bertani agar). Seven predominant isolates were screened using Sudan Black B staining. Positive isolates underwent secondary screening on PHB-selective media. Morphological and biochemical characterizations were conducted, followed by 16S rRNA (ribosomal Ribonucleic Acid) gene sequencing for molecular identification. Out of seven isolates, two tested positive for PHB production. Isolate KG1 showed 95% similarity to *Enterococcus faecium* [DSM 20477], while KG6 had 100% similarity to *Neobacillus niacini* [NBRC 15566]. The study highlights the potential of indigenous soil bacteria in PHB production, offering promising candidates for sustainable bioplastic development and reducing dependence on petroleum-based plastics.

Introduction

Plastic materials widely used in our day-to-day activities are non-biodegradable and pose environmental hazards. Synthetically generated polymers are readily available and cost-effective, but their widespread use has consequential ecological impacts. Due to this, millions of plastic items are accumulating in the environment, leading to pollution. Hence, there is an urgent need to replace the non-biodegradable plastics with naturally occurring biodegradable polymers. Polyhydroxybutyrate (PHB) is

one such polymer produced by bacteria in response to environmental and nutritional stress, and it is stored as a reserve food material in inclusion bodies. Being in the environment for a long time leads to the release of toxic chemicals from complex plastics. Biopolymers offer an 'eco-friendly' perspective in nature, protecting the environment from hazards caused by synthetic plastics. Biodegradable polymers are synthesized from various natural sources, such as microorganisms, plants, and animals. Polyhydroxyalkanoate (PHA), a naturally



biodegradable macromolecule, has been shown to mitigate the negative effects of petroleum-based plastic waste in the environment. When PHB is associated with other polymers, the polymer characteristics, such as thermal properties, crystallinity, tensile strength, elasticity, and ductility, have been shown to be improved (Villalobos et al., 2016). Figure 1 illustrates a graphical understanding of PHB degradation in soil. Due to its superior biodegradability and biocompatibility, PHB, a semi-crystalline polymer of microbial origin, has emerged as one of the most intriguing biomaterials. These bacteria produce PHA when there is excess carbon as well as low nitrogen content and the PHA could be degraded into water and carbon dioxide by some environmental microorganisms, unlike the synthetic plastics that remain in the environment for long after their intended use (Musa et al., 2016). PHBs are microbially produced biopolyester molecules that are intracellularly accumulated as granules by various microbes under nutritional and environmental stress conditions (Carlozzi et al., 2020). The size of these biogenic granules' ranges between 0.2 and 0.5 μm and they are stored in the cytoplasm in the form of insoluble inclusion bodies (Adebajo et al., 2024). The first kind of PHA to be identified and extensively studied is polyhydroxybutyrate (PHB). PHB is similar to synthetic petrochemical polymers, with features such as biocompatibility and biodegradability. The production of microbial PHB can be regulated by alteration of the growth parameters such as pH, temperature, incubation time and salinity (Mahato et al., 2021; Kate et al., 2019).



Figure 1. Graphical abstract Biodegradation of polyhydroxybutyrate films in soil

[Sources:

https://www.researchgate.net/publication/304068716_Biodegradation_of_different_formulations_of_polyhydroxybutyrate_films_in_soil#fullTextFileContent [accessed Mar 18 2025].

Materials and Methods

Soil Sample Collection

Soil samples were collected from various sites in the Mehsana and Bhavnagar districts, including the Cowshed

area, dairy industry, and dumping sites (Table 1). Soil samples were collected in a sterile zip-lock bag. Samples were transported to the laboratory for analysis and stored at 4°C until processing.

Enumeration of Bacterial Culture

The typical serial dilution protocol was followed for screening the predominant cultivable bacterial isolates from stored soil samples after thawing at room temperature. The spread plate method was followed to inoculate approximately 50 μl of 10^{-6} dilutions of collected soil samples, on sterile LB agar plates, and were properly labelled. Triplicate plates inoculated from each sample and kept under incubation for 48 h at 37 °C along with a sterile control. The predominant and individual colonies were isolated from the master plate and sub-cultured to obtain pure colonies by streaking and conserved at 4 °C for further analysis. Bacterial characterization was conducted using cultural, morphological, and biochemical methods in accordance with Bergey's Manual (Musa et al., 2016).

Screening of bacterial isolates for polyhydroxybutyrate (PHB) production

Primary screening for PHB-producing bacteria

Around 7 predominant bacterial isolates were selected based on their active growth on LB agar plates, and the colonies were tested with the Sudan Black B stain as per the protocol of Obruča et al. (2022) to assess their PHB-producing potential. Fresh isolated bacterial strains were each inoculated into the sterile nutrient agar plates containing 1% glucose and the plates were incubated at 30°C for 24 hours. Concisely, 2 mL of Sudan Black B stain (0.05%) was poured over the well-grown colonies on the LB plate and incubated at room temperature for 30 min. The plates were then destained by washing with ethanol (96%) to remove excess stain from the colonies. The isolates that retained their black or dark blue color after destaining were attributed as proficient PHB-producing strains (Dhanial et al., 2023). Subsequently, the stained culture plates were again incubated for 30 min, and noticed the color changes were observed as dark greenish-blue, considered as PHB positive (Dhanial et al., 2023) as primary screening.

Secondary screening for PHB-producing bacterial isolates

Out of Seven, only two isolates showed positive for primary screening and later which was subjected to the secondary screening on PHB selective media (containing 2.5 g, 25 g, 100 g, 20 g, 1 g, 100 g, 10 g, 1.2 g, 20 g L of $\text{H}_2\text{K}_2\text{O}_4\text{P}$, $\text{HNa}_2\text{O}_4\text{P}$, Mannitol, NaCl, MgSO_4 , $\text{C}_3\text{H}_3\text{NaO}_3$, Peptone, Bromothymol blue, and agar respectively) as per the procedure of (Narayanan et al., 2020). The culture inoculated plates were incubated for three days at 35°C,

and bluish colonies were observed and confirming that the test isolates possess PHB synthesizing potential (Narayanan et al., 2020).

Biochemical and Molecular identification of potential PHB producers and their phylogenetic analysis

The isolate was characterized morphologically by observing the standard microbiological markers (Gram reaction, motility and spore formation). The biochemical characterization of the isolates was performed using a series of tests, including the MRVP test (Methyl Red–Voges Proskauer test), the Citrate Utilization test, and the catalase test. The qualitative screening for PHB was performed by growing the cultures on PHB-specific medium and staining them with Sudan black B (Krishnan et al., 2017).

Molecular Identification of PHB-Accumulating Bacteria by 16S rRNA Gene Analysis

To isolate kg1 and kg6, their genomic DNA (Deoxyribonucleic Acid) was extracted and utilized for PCR (Polymerase Chain Reaction) amplification of the 16S rRNA gene.

1. DNA was isolated from the culture. The quality of DNA was evaluated on a 1.0% Agarose Gel, and a single band of high-molecular-weight DNA was observed.

2. PCR amplified fragments of the 16S rRNA gene. A single discrete PCR amplicon band was observed upon agarose gel resolution. The PCR amplicon was purified using a column to remove contaminants.

4. DNA sequencing reaction of PCR amplicon was carried out with 27F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The 16S rRNA sequence was used to perform BLAST (Basic Local Alignment Search Tool) against the NCBI (National Center for Biotechnology Information) GenBank database.

To determine the taxonomic position of the isolates, a phylogenetic tree was constructed in MEGA11 using a neighbor-joining algorithm with the Jukes–Cantor distance estimation method, and bootstrap analyses were performed with 1000 replicates. This analysis involved 11 nucleotide sequences.

Results

Screening for PHB-Producing Bacteria

In the present study, we are exploiting the isolation and identification of microbial strains from the collected soil samples of the North and West Gujarat districts and which could be used later successfully for PHB production.

Table 1. Soil samples from various sites of Mehsana and Bhavnagar districts of North Gujarat were collected.

Sl No.	Sample Name	Collection Area
1	Soil from Cowshed area	Mehsana
2	Soil from Cowshed area	Rojiya, Bhavnagar
3	Soil from domestic waste landfill	Mehsana
4	Soil from Dairy industry (Dudhsagar Dairy)	Mehsana
5	Soil of crop yielding farm	Rojiya, Bhavnagar
6	Soil of the microfo-rest of Ganpat university	Ganpat University, Mehsana

Isolates on PHB media (kg1 and kg6)

The isolated samples were screened for PHB production on PHB-specific media. Based on the results, of the seven (7) isolates grown on Nutrient agar, two (2) produced PHB, as shown in Figure 3. Further, the confirmation was carried out by isolating the characterization.

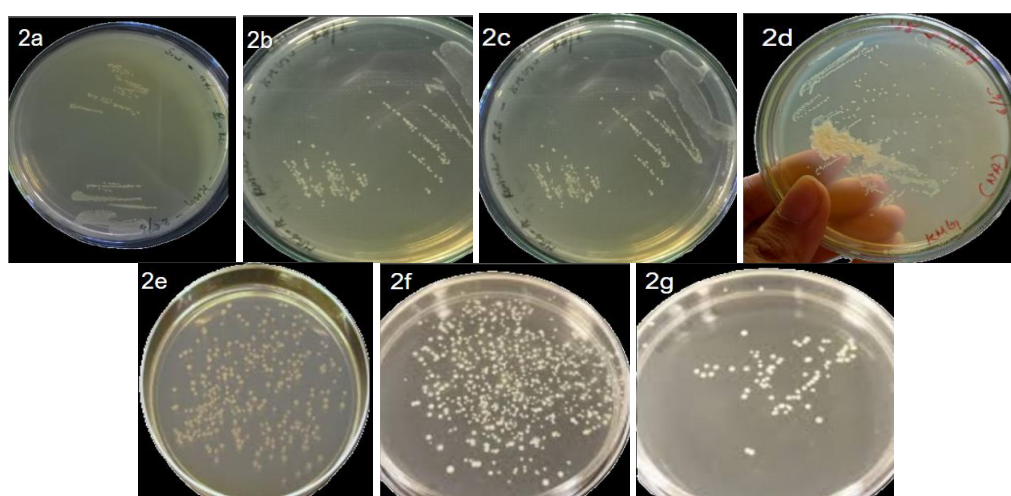


Figure 2. Nutrient Agar plates. Figure shows the images of isolates from the collected soil sample. Isolates were selected based on active growth on LB agar plates, and the colonies were tested with the Sudan Black B stain as per the protocol of Rendón-Villalobos et al. (2016) to assess their PHB-forming potential.

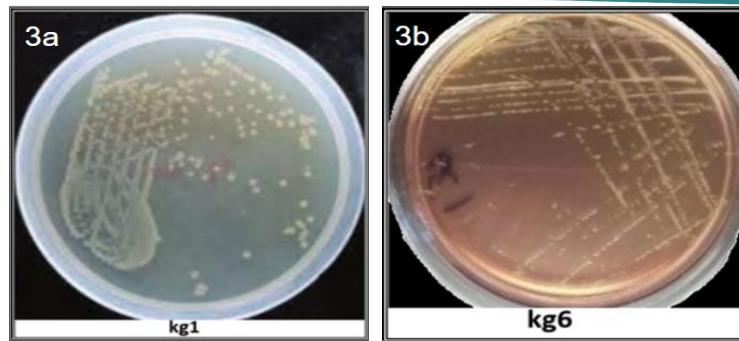


Figure 3. PHB production on PHB-specific media. The isolated samples were screened for PHB production on PHB-specific media, 2 isolates (kg1 and kg6) showed the ability to produce PHB.

Phylogenetic Tree:

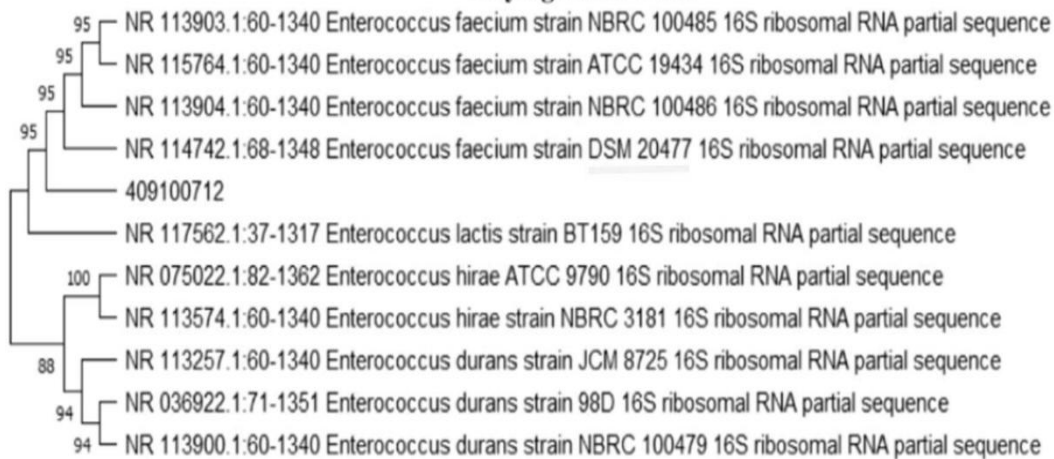


Figure 4. Molecular Phylogenetic analysis of kg1 by the Neighbor-Joining method. Phylogenetic tree of kg1 isolates, which have the highest homology with strain DSM 20477. The phylogenetic tree was constructed using the neighbor-joining method with 100 bootstrap replicates.

Phylogenetic Tree:

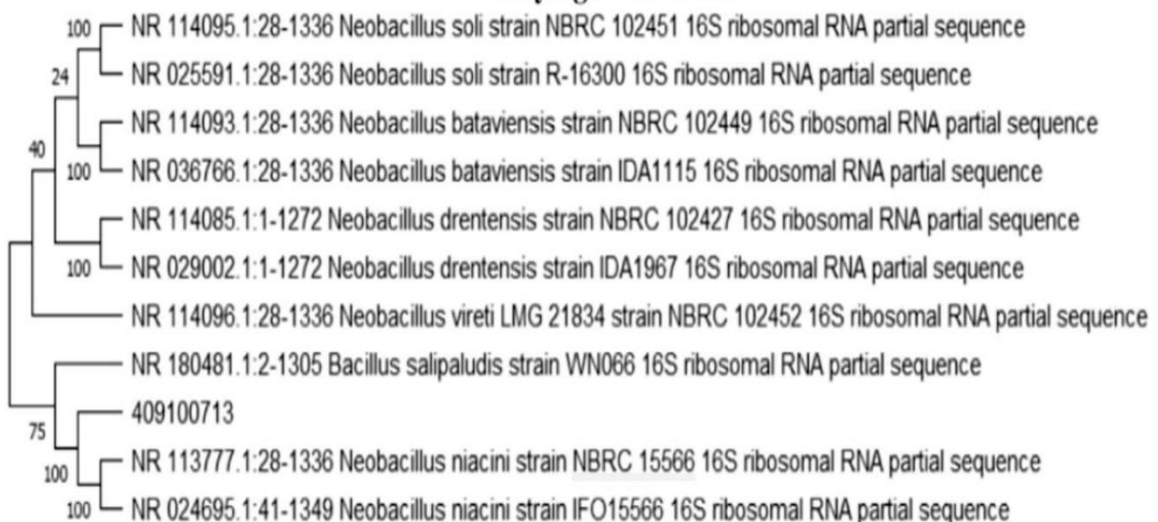


Figure 5. Molecular phylogenetic analysis of kg6 using the Neighbor-Joining method. Phylogenetic tree of kg6 isolates, which have the highest homology with strain NBRC 15566. The phylogenetic tree was constructed using the neighbor-joining method with 100 bootstrap replicates.

Table 2. Morphological and Biochemical characteristics of isolates. The table summarizes the key morphological and biochemical characteristics used to identify and differentiate bacterial isolates.

Isolates	kg ¹	kg ⁶
Gram reaction	+ve	+ve
Shape	Cocci	Rod shape
Catalase test	-ve	+ve
Citrate Utilization	-ve	+ve
MR – VP test	+ve	+ve
'+' indicates a positive result; '-' indicates a negative result		

Morphology and biochemical characterization of the kg¹ and kg⁶

The PHB-producing isolate was determined based on morphological and biochemical characteristics as depicted in Table 2.

The PHB production isolate (kg¹ and kg⁶) was identified by comparative 16S rRNA gene-sequencing analysis. The alignment results show that the 16S rRNA sequences of the isolated strains kg¹ and kg⁶ were highly homologous with 95% similarity to *Enterococcus faecium* [DSM 20477, Deutsche Sammlung von Mikroorganismen] and 100% similarity with *Neobacillus niacini* [NBRC 15566, National Institute of Technology and Evaluation (NITE) and Biological Resource Center] (Figure 4 & 5).

Staining

PHB production by the bacterial isolates was assessed using staining methods such as Gram's and Sudan black, as depicted in Table 3.

Table 3. Gram staining and Sudan staining results. This table presents the results of Gram and Sudan staining for microbial isolates. Gram staining differentiates bacteria based on cell wall composition: Gram-positive (G⁺) organisms retain the crystal violet stain (purple), while Gram-negative (G⁻) do not (pink/red). Sudan staining detects lipid inclusions: a positive result indicates the presence of lipid-rich granules stained red by Sudan dye, whereas a negative result indicates their absence.

Sample	Organism	Gram staining	Sudan staining
Isolate 1	<i>Enterococcus faecium</i>	+ve	+ve
Isolate 2	<i>Neobacillus niacini</i>	+ve	+ve
'+' indicates a positive result; '-' indicates a negative result			

a. Gram staining

Gram staining was carried out, and the isolates were found to be Gram-positive as shown in figure 6.

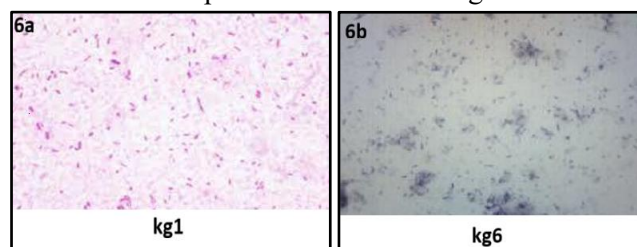


Figure 6(a&b). Gram Staining of PHB producing isolates was performed. (6a): kg¹ isolate identified as gram +ve; (6b) kg⁶ isolate identified as gram +ve.

b. Sudan staining

Isolates from PHB-specific Agar medium were picked for further screening using Sudan black solution, as shown in Figure 7.

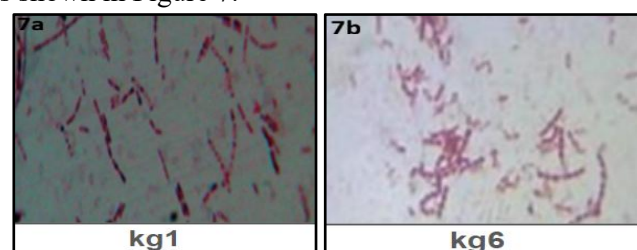


Figure 7 (a&b). Sudan Black B staining of PHB-producing bacterial isolates. (7a): kg¹ isolate showing positive staining (Sudan +ve), indicating PHB accumulation. (7b): kg⁶ isolate showing positive staining (Sudan +ve), indicating PHB accumulation.

Biochemical Tests

Biochemical tests, such as Methyl Red and Voges-Proskauer, Citrate Utilization test, and Catalase test, were performed on selected PHB-producing bacteria, namely kg¹ and kg⁶, to further characterize them. The result is depicted in figure 8.

16sRNA sequencing

The 16S rRNA gene sequences and phylogenetic analysis were considered powerful tools for identifying bacterial isolates.

kg¹ – *Enterococcus faecium*

The phylogenetic tree indicates that kg¹ is found to be *Enterococcus faecium* based on nucleotide homology analysis, as illustrated in Figure 4.

kg⁶ – *Neobacillus niacini*

The phylogenetic tree indicates that kg⁶ is found to be *Neobacillus niacini*, based on nucleotide homology analysis, as shown in figure 5. Based on the maximum identity score, the first 10 sequences were selected and aligned using multiple-sequence alignment software.

#kg¹ is found to be *Enterococcus faecium* based on nucleotide homology analysis & phylogenetic analysis.

Isolate 1 is closely related to the type strain *Enterococcus faecium* [DSM 20477].

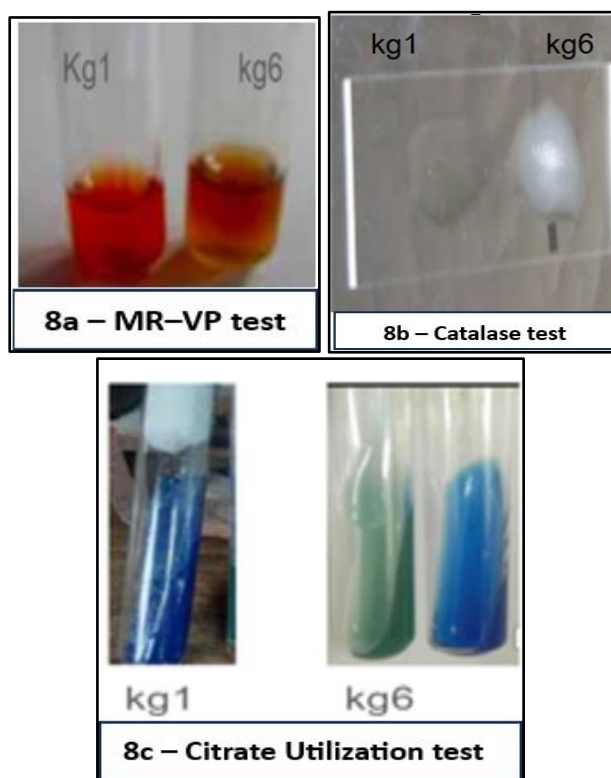


Figure 7. Biochemical tests such as Methyl Red–Voges Proskauer (MR–VP) test, Catalase test, and Citrate Utilization test of identified isolates kg1 and kg2 [MR–VP test: kg1 - "+" = Positive reaction; kg6 - "+" = Positive reaction; Catalase test: kg1 - "-" = Negative reaction; kg6 - "+" = Positive reaction; Citrate Utilization test - "-" = Negative reaction; kg6 - "+" = Positive reaction].

#kg6 is found to be *Neobacillus niacini* based on nucleotide homology analysis & phylogenetic analysis. Isolate 2 is closely related to type strain *Neobacillus niacini* [NBRC 15566].

The PHB-producing isolates kg1 and kg6 were identified by comparative 16S rRNA gene-sequencing analysis. The alignment results show that the 16SrRNA sequences of the strain kg1 and kg6 were highly homologous with 95% and 100% similarity to *Enterococcus faecium* [DSM 20477] and *Neobacillus niacini* [NBRC 15566], respectively. The gene sequence obtained from isolates kg1 and kg6 was submitted to GenBank and assigned the accession number. A comparison of partial 16S rDNA sequences was performed against the National Center for Biotechnology Information's (NCBI) collection of 16S rDNA sequences in public databases. Phylogenetic trees were constructed for isolated species using the neighbor-joining method with MEGA software.

Discussion

The present study focused on the isolation and characterization of bacterial strains capable of producing polyhydroxybutyrate (PHB), a biodegradable polymer with significant potential as an alternative to conventional plastics (Musa et al., 2016; Krishnan et al., 2017; Sinha, 2017; Majumder et al., 2020). Synthetic plastics derived from petrochemicals persist in the environment due to their non-biodegradable nature, contributing to long-term ecological degradation. As one solution to this problem, polyhydroxyalkanoates (PHA) have attracted attention as a biomass-based polymer made from non-edible materials (Nishida et al., 2018; Mostafa et al., 2020). Of the seven isolates initially grown on nutrient agar, two strains, viz., KG1 and KG6, demonstrated the ability to produce PHB on PHB-specific media. This preliminary screening was an essential step in narrowing down potential PHB producers among the collected soil samples from North and West Gujarat.

Morphological and biochemical characterization of the PHB-producing isolates provided foundational insight into their identity. Gram staining revealed that both isolates were Gram-positive, a trait consistent with many known PHB-producing genera, such as *Bacillus* and *Enterococcus*. Further confirmation of PHB production was achieved through Sudan Black B staining, which clearly demonstrated the presence of intracellular PHB granules. Sudan Black B is a lipophilic dye known for its specificity in staining PHB, supporting its utility in rapid phenotypic screening (Akaraonye et al., 2010). Positive isolates obtained from solid medium screening were detected for the presence of PHA granules using a microscope (Jasmine et al., 2021; Dienye et al., 2023).

Biochemical tests further supported the differentiation and identification of the isolates. Results from the Methyl Red (MR), Voges–Proskauer (VP), Citrate utilization and Catalase tests offered additional characterization traits useful for preliminary taxonomic placement. These tests are traditionally used to identify metabolic capabilities and enzymatic properties, providing insight into the physiological traits of the bacteria, which often correlate with their ecological niches and functional roles (Kate et al., 2019).

To achieve definitive identification, molecular characterization was conducted using 16S rRNA gene sequencing—a widely accepted tool in bacterial taxonomy due to its high degree of conservation and informativeness. Phylogenetic analysis based on the neighbor-joining method revealed that isolate KG1 shared 95% sequence homology with *Enterococcus faecium* [DSM 20477], while isolate KG6 demonstrated 100% similarity with

Neobacillus niacini [NBRC 15566]. These findings are significant as both *Enterococcus* and *Neobacillus* genera have been previously reported to harbor strains capable of producing PHB, particularly under nutrient-limiting conditions that favor PHB accumulation as a carbon and energy reserve (Musa et al., 2016; Obruča et al., 2022).

The identification of KG1 as *Enterococcus faecium* is particularly notable, as this species is more commonly associated with clinical or gut environments. Its ability to produce PHB may suggest an adaptive mechanism for surviving harsh or nutrient-depleted environments. On the other hand, KG6, identified as *Neobacillus niacini*, aligns more closely with typical soil bacteria known for spore formation and PHB accumulation. The 100% sequence homology also strengthens confidence in its identification and potential utility for industrial PHB production (Musa et al., 2016; Obruča et al., 2022).

The gene sequences for both isolates have been submitted to GenBank, ensuring accessibility for future comparative studies. The phylogenetic trees (Figures 7 and 8) clearly illustrate the evolutionary relationship of these isolates to their respective type strains, validating the molecular data and aligning with previous literature on PHB-producing microbes (Narayanan et al., 2020; Krishnan et al., 2017).

In conclusion, this study describes the potential of isolates for PHAs, their uses, the various attempts towards the production of PHAs, focusing on the utilization of cheap substrates and further the development of different fermentation strategies for the production of these polymers for future studies (Akaraonye et al., 2010). The study successfully identified two novel PHB-producing bacterial isolates, KG1 (*Enterococcus faecium*) and KG6 (*Neobacillus niacini*), using an integrated approach that combined phenotypic screening, biochemical testing, and molecular analysis. These findings expand the spectrum of known PHB-producing bacteria and offer promising candidates for future biopolymer production, especially under optimized fermentation conditions. Further studies focusing on yield optimization, substrate utilization, and genetic engineering may unlock their full industrial potential.

Conclusion

In conclusion, the present study successfully isolated and characterized two bacterial strains, *Enterococcus faecium* (KG1) and *Neobacillus niacini* (KG6), capable of producing polyhydroxybutyrate (PHB), a biodegradable alternative to petrochemical plastics. Through morphological, biochemical and molecular analyses, both isolates were confirmed as efficient PHB producers. *E.*

faecium displayed adaptability to nutrient-limited environments, while *N. niacini* showed strong potential typical of soil-dwelling, spore-forming PHB producers. Molecular identification via 16S rRNA sequencing and phylogenetic analysis validated their taxonomic placement, and the gene sequences were submitted to GenBank for future reference. These findings broaden the diversity of known PHB-producing bacteria and highlight their potential for sustainable biopolymer production. Future research should focus on optimizing culture conditions, utilizing cost-effective substrates, and exploring genetic modifications to enhance PHB yield for industrial-scale applications.

Conflicting interest

The authors have no conflict of interest to declare.

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